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# TECHNICAL MANUSCRIPT 99

# SUSCEPTIBILITY OF SOOTY TERNS TO VENEZUELAN EQUINE ENCEPHALITIS (VEE) VIRUS

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# SUSCEPTIBILITY OF SOOTY TERNS TO VENEZUELAN EQUINE ENCEPHALITIS (VEE) VIRUS

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## ABSTRACT

Sooty terns were found to be highly susceptible to infection with Venezuelan equine encephalitis virus by the respiratory route. These birds developed viremia to high titers, but without obvious clinical signs of infection. Further, by the fifth day after exposure to virus the viremia had declined to negligible levels. A bird-mosquito-bird transmission cycle was demonstrated with Aedes triseriatus mosquitoes and sooty terns. A bird-bird transmission cycle was also suggested by infections in control birds held in the same area as infected birds.

# I. INTRODUCTION

There have been many reports of isolations of arthropod-borne virus from the tissues of wild birds. Eastern equine encephalitis (EEE) virus was isolated from ring-necked pheasants[1] and sparrows,[2] among others. Further, in tests of susceptibility, EEE virus was found to be infective for white ibis, American egret, snowy egret, purple grackle, red-winged blackbird, cardinal, sparrow and cedar waxwing[3] whether infected subcutaneously or by mosquito bite. Tests by Hammon et al[4] with the virus of Western equine encephalitis have shown that English sparrows, house finches, tricolored red-winged blackbirds, and white-crowned sparrows develop viremia after subcutaneous inoculation. It was concluded that wild birds could be excellent sources for mosquito infection. The susceptibility of cardinals, white-throated sparrows, English sparrows, pigeons, and mourning doves to Venezuelan equine encephalitis (VEE) virus was reported by Chamberlain et al.[5] Mosquito transmission was demonstrated in this latter effort.

All of these studies support the concept that wild birds may be an important reservoir of arboviruses. The intermediate host from bird to bird or bird to man has been assumed to be the mosquito. Indeed, a number of studies have verified the efficiency of mosquitoes for transmission, including those of Chamberlain et al.[5]

The studies reported here indicate the possibility of a bird-to-bird cycle without an intermediate mosquito host. Wild birds, in this case sooty terns, were tested for susceptibility to aerosols of VEE virus employing quantitative methods. Further, data on transmission of infectious agent between birds was obtained both with and without mosquito vectors.

# II. MATERIALS AND METHODS

## A. BIRDS

The birds employed in this study were sooty terns (Sterna fuscata oahuensis). Two hundred and fifty-seven of the species were captured in the wild state and provided to these laboratories for testing.*

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* In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

Because of large numbers of nonspecific deaths during the testing period, only 135 were maintained for a sufficient period to provide test data. Upon receipt the birds were placed uncaged in a large room. The maximum holding time was about four weeks. At the time of test, the birds were transferred to wooden wire-front cages that provided one square foot of floor space per bird. These cages were placed within ventilated cabinets[a] for the required holding periods. The birds were fed chopped squid twice a day. In some instances it was necessary to force-feed and in these cases whole smelt were used.

## B. VIRUS

The Trinidad strain of VEE virus grown in embryonated eggs was employed in these studies. To control bacterial contamination, 1000 units of Penicillin G, 1000 micrograms of dihydrostreptomycin, and 200 micrograms of acromycin were added per milliliter of the harvested viral suspension. The virus in aerosol samples was assessed by inoculating intracerebrally 0.03 milliliter of appropriate dilutions of the collecting fluid into 10- to 12-gram Swiss-Webster mice. Virus was diluted in beef heart infusion broth (Difco) and samples were tested in duplicate for virus concentration. Eight mice were inoculated per dilution. Mouse deaths occurring during the period 72 hours through ten days after inoculation were considered to be specific.

## C. VIREMIA

The level of viremia in each bird was determined on the second, third, fourth, and fifth days after challenge. Controls were bled on the same days. On each day, one-tenth milliliter of blood was collected from the median vein of the wing and diluted in 9.9 milliliters of phosphate-buffered saline to yield a 1:100 dilution. Further dilutions were made to $10^{-5}$ and eight mice were inoculated intraperitoneally with 0.5 milliliter from each dilution.

## D. SERUM NEUTRALIZATION TESTS

Before challenge, and again 21 days after challenge, blood was collected from the birds for serum neutralization tests. In these tests, dilutions of virus included the estimated end point, one dilution above, and two dilutions below. The bird sera were not inactivated before conducting the neutralization tests. Eight mice were inoculated IC with 0.03 milliliter from each dilution. SN results are reported as logarithms of virus neutralized.

## E. AEROSOL FACILITIES

Virus aerosols were produced in chambers similar to those described by Wolfe.[7] The temperature was 85°F; the relative humidity was maintained at 85 per cent. Samples of the aerosol for estimating virus concentrations were collected by liquid impingers similar to those described by Tyler et al.[8] When it was desirable to expose birds to graded respiratory doses, the aerosol was allowed to age until natural decay of the virus reduced concentrations to the desired levels. Aerosols were produced by a nozzle system capable of producing a cloud with a particle mass median diameter of about four microns. Birds were exposed to aerosols by inserting their heads into the cloud chamber through slits in rubber diaphragms mounted in the chamber wall. During the exposure the birds' bodies were confined in small boxes. The amount of virus inhaled was estimated by Guyton's equation for respiratory volume.[9]

## F. VECTOR TRANSMISSION

Female Aedes aegypti and Aedes triseriatus mosquitoes were allowed to feed on viremic terns. Subsequently they were allowed to feed on normal terns and then each insect was triturated and assessed for virus content. Mosquitoes were triturated in 1.0 milliliter of phosphate-buffered saline containing 100 units of penicillin per milliliter and 100 micrograms of streptomycin per milliliter. Each of five mice was inoculated with 0.03 milliliter of the triturate. Deaths of all mice during the period of 72 hours through ten days after inoculation were considered to be due to VEE virus infection.

Mosquitoes were held in ice-cream cartons with marquisette covers over an open end. Insects were fed daily by placing fresh cotton pads saturated with ten per cent sucrose on the cage covers, but food was not provided for a period of 24 hours prior to feeding experiments.

# III. RESULTS

## A. VIREMIA, MORTALITY, AND PATHOLOGY

Studies were made of viremia responses in sooty terns after exposure to high concentrations of inhaled virus (greater than 5000 $MICLD_{50}$'s inhaled). Because of the high proportion of responses, additional studies were conducted using decreasing dose levels. These were, in Phase II, 2600, 1650, and 66 $MICLD_{50}$ units inhaled and in Phase III, 39 and less than

one $MICLD_{50}$ units inhaled. The proportion of birds responding, however, remained constant regardless of challenge dose. The results obtained in the three phases are presented in Table I. It should be noted that in Phase II, birds were only screened for viremia by the inoculation into mice of $2 \times 10^{-2}$ and $2 \times 10^{-3}$ dilutions. In Phases I and III, virus end points were achieved. The data in general are consistent over all phases with respect to viremic patterns. The highest level was usually found in the first blood sample (day 2 after exposure) and then generally decreased through Day 5. There was also a decrease with time in the per cent infected, indicating that infections were being cleared. Note that in Phase II, higher percentages of birds were infected throughout the period of study. It was also found that many of these birds failed to exhibit viremia until later than the second day after exposure. These results are discussed later in relation to infection in control birds.

It appears from Phase I that infections in birds were fatal. Reference is made to the decrease in number of birds tested throughout five days. For example, on the second day after exposure, 22 birds were alive, but on Day 5 only six were alive. However, in Phase II, 47 of 52 birds and in Phase III, 11 of 12 birds survived the period of viremia. As indicated previously, nonspecific deaths were occurring sporadically in birds whether they were in holding situations or actually in test. Necropsy and histopathologic examinations were performed on birds that died during the course of infection and during holding under clean conditions. Comparisons of these results showed no consistent pathologic differences. Further, there were no signs in infected birds of pathologic changes noted in other studies with pheasants infected with VEE virus.*

Figure 1 is a graphical representation of the average viremic pattern obtained in Phases I and III. These data are consistent with those presented by Chamberlain in his studies.[5] Further, it is apparent that the viremic response was independent of the dose level producing it.

The control birds in this study contributed decisively to the conclusions to be drawn. Three types of controls were employed. Type I: exposure-line controls were those birds that underwent all handling procedures except actual exposure. This included confinement during incubation in cages adjacent to exposed birds. Type II: individuals not taken to the exposure area but held in cages adjacent to those of exposed birds. Type III: birds handled similarly to those in Type II but held in cages with exposed birds. Control results are summarized in Table II. Data obtained in the three phases were combined because of similar results.

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* Hirth, R.S., U.S. Army Biological Laboratories; Personal communication of 18 April 1963.

TABLE I. RESULTS OF VIREMIA TESTS IN SOOTY TERNS EXPOSED TO AEROSOLS OF VEE VIRUS[a/]

| Days Post-Exposure[b/] | Number of Birds Tested[b/] | Positive Viremia, per cent | Range of Viremia, log $MIPLD_{50}$/ml of blood |
|---|---|---|---|
| | Phase I (>5000 $MICLD_{50}$ inhaled) | | |
| 2 | 22 | 68.2 | 2.8 - 4.8 |
| 3 | 21 | 68.2 | 2.3 - 4.8 |
| 4 | 19 | 47.0 | 2.3 - 3.8 |
| 5 | 6 | 0.0 | 0.0 |
| | Phase II (26,000, 1650, or 66 $MICLD_{50}$ inhaled)[c/] | | |
| 2 | 52 | 94.2 | 2.3 - >3.8 |
| 3 | 50 | 94.0 | 2.3 - >3.8 |
| 4 | 49 | 77.6 | 2.3 - >3.8 |
| 5 | 47 | 61.7 | 2.3 - >3.8 |
| | Phase III (39 or <1 $MICLD_{50}$ inhaled)[c/] | | |
| 2 | 12 | 66.6 | 3.3 - 5.3 |
| 3 | 12 | 58.3 | 2.8 - 4.8 |
| 4 | 12 | 58.3 | 2.3 - 5.6 |
| 5 | 11 | 27.7 | 2.3 - 2.8 |

a. Aerosol Conditions: 85 per cent relative humidity, 85°F.
b. The column headed "Days Post-Exposure" indicates days on which blood samples were taken for tests for viremia; the column headed "Number of Birds Tested" indicates the number of birds that had survived and were therefore bled on the indicated days.
c. Responses totaled over dose groups because of similar results in each group.

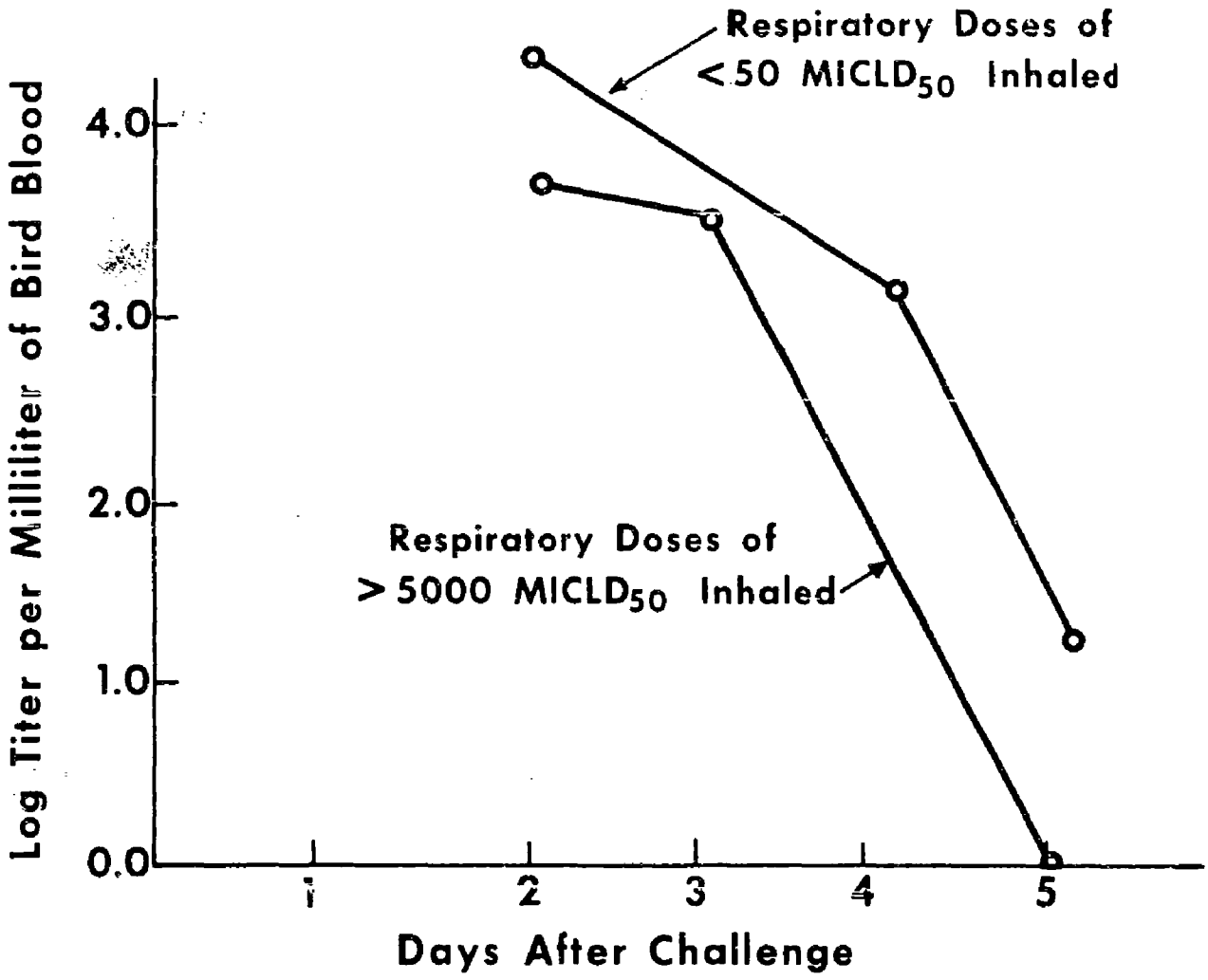


Figure 1. Viremia as a Function of Time in Sooty Terns.

TABLE II. RESULTS OF TESTS FOR VIREMIA IN CONTROL GROUPS OF SOOTY TERNS

| Control Type | Number Tested | Number Viremic | First Day of Viremia Post-Exposure | Range of Viremia, log $MIPLD_{50}$/ml of blood |
|---|---|---|---|---|
| Exposure-line control | 8 | 5 | 2,2,2,4,5 | 2.6 to >3.5 |
| Holding-line control | 23 | 8 | 2,5,5,5,5,5,5,4 | 2.0 to >3.5 |
| Cagemate control | 6 | 2 | 3,5 | 4.0 to 5.4 |

Many cases of viremia were detected from these control birds. There was, however, a pattern associated with the type of control as regards the day on which viremia was first detected. With the exposure-line controls (Type I), viremias were at high levels as early as in the exposed birds. Previous experience has indicated that extremely low levels of contamination can exist in the exposure cabinets adjacent to the aerosol chamber. It appeared that these levels were sufficient to infect some control birds and produce in them viremias identical to those of birds receiving massive doses. These results are consistent with the results obtained in planned exposures to extremely low inhaled doses.

The results obtained with the holding-line controls (Type II) indicate that the first day of viremia occurred, in most cases, about the fifth day after exposure of their holding-area mates. This suggested that the infective doses for the holding-line controls came from the infected birds. It appears that an infected bird-to-normal-bird transmission was occurring, and it is believed that these were aerosol infections. It should be noted that in Phase III, the highest initial dose of 39 $MICLD_{50}$ units inhaled, a concentration far higher than could have occurred in the exposure cabinet, initiated a cycle in which almost half of the total exposed and control birds developed viremia.

B. SEROLOGY

Serum neutralization (SN) titers were determined on sera obtained from birds before exposure and on sera collected from survivors 21 days after exposure in Phases I and II. Tests for each (before and after exposure) were conducted concurrently. The results obtained before exposure are presented in Table III.

TABLE III. EFFECT OF PRE-EXPOSURE SN ON SOOTY TERN RESPONSES TO AEROSOLS[a] OF VEE

| SN Index, pre-exposure | Number of Birds | Per Cent of Total | Number of Birds Viremic After Exposure | Per Cent Positive Viremia Within SN Group |
|---|---|---|---|---|
| 0.0 - 0.5 | 3 | 4 | 3 | 100 |
| 0.6 - 1.0 | 11 | 15 | 10 | 91 |
| 1.1 - 1.5 | 14 | 19 | 12 | 86 |
| 1.6 - 2.0 | 31 | 42 | 30 | 97 |
| 2.1 - 2.5 | 11 | 15 | 10 | 91 |
| >2.5 | 4 | 5 | 3 | 75 |

a. Doses: >5000, 1650, or 66 $MICLD_{50}$ inhaled; responses were independent of dose.

TABLE IV. EFFECT OF AEROSOL EXPOSURE ON SN INDEX OF A SELECTED GROUP[a] OF SOOTY TERNS

| Inhaled Dose $MICLD_{50}$ | Number of Birds | Number of Birds Viremic | Post-Exposure Index |
|---|---|---|---|
| >5000 | 5 | 5 | 1, 1.6; 4, $\geq$2.5 |
| 1650 | 1 | 1 | 2.6 |
| 66 | 2 | 2 | $\geq$2.3 |
| Control | 6 | 0 | all <0.9 |

a. Sooty terns from Phases I and II exhibiting pre-exposure SN index of 1.0 or less and surviving 21 days post-exposure. Control birds having pre-exposure SN index of 1.0 or less, no exposure, no evidence of viremia, and surviving 21 days from first bleeding.

Forty-two per cent of the bird sera obtained prior to VEE virus exposure exhibited log indices in the range of 1.6 to 2.0. There was, however, no apparent effect of these indices on subsequent responses to inhaled virus. For example, 91 per cent of the birds with pre-exposure indices of 2.1 to 2.5 exhibited viremia after exposure. Because of these unexpectedly high indices, significant rises in index (1.8 logs) could not, in many cases, be detected with the dilutions employed. The results in Table IV, however, indicate the effect of exposure on SN index for birds that had a pre-exposure index of less than one. These are presented as a function of dose-level group and control. A significant rise in index was obtained with all exposed birds. All of these were viremic following challenge. Six control birds in the study had initially low indices and did not develop viremia. Twenty-one days after the first bleeding, there was no rise in index. These results confirmed the specificity of the tests for viremia and further tended to confirm the thought advanced earlier that VEE virus infections are not fatal for sooty terns.

## C. MOSQUITO TRANSMISSION

Ten sooty terns were exposed to aerosols of VEE virus to obtain hosts for vector transmission tests. Groups of 50 Aedes aegypti or Aedes triseriatus were allowed to take a blood meal from each of the seven survivors among this group 48 hours after challenge. The birds were tested for viremia at that time and four of the seven had viremia (greater than $10^5$ $MICLD_{50}$ units per milliliter of blood). The potentially infected mosquitoes were held for 21 days (extrinsic incubation period). Fifteen insects from each group of 50 were starved for 24 hours and then allowed to refeed on normal terns. The mosquitoes were triturated after this feeding. Terns were tested for viremia on the second, third, fourth, and fifth days after being bitten. Positive transmission of VEE virus to sooty terns by A. triseriatus mosquitoes was obtained when it could be shown that the initial blood meal was from an infected bird. A. aegypti, however, failed to transmit the virus. Triturations of a test group of mosquitoes indicated that only one of 46 A. aegypti was actually infected, although 36 of 47 A. triseriatus were infected 21 days after feeding on infected birds. Results of transmission tests are shown in Table V.

TABLE V. TRANSMISSION OF VEE VIRUS BY MOSQUITOES TO SOOTY TERNS

| Presence of Virus in Initial Blood Meal[a] | Species | Total Number of Bites Received by Uninfected Terns[b] | Number of Bites From Confirmed Infected Mosquitoes | Terns Developing Viremia |
|---|---|---|---|---|
| - | A. triseriatus | 7 | 0 | - |
| + | A. aegypti | 14 | 1 | - |
| + | A. triseriatus | 8 | 8 | + |
| - | A. aegypti | 12 | 1 | - |
| + | A. triseriatus | 4 | 4 | + |
| + | A. aegypti | 12 | 1 | - |
| - | A. triseriatus | 11 | 0 | - |

a. + indicates a viremia $<10^{5}$ $MIPLD_{50}$'s/ml bird blood.
- indicates no viremia in terns at $2 \times 10^{-2}$ dilution of blood.

b. Number of bites received was based on presence of blood in the 15 mosquitoes allowed to feed on a single tern.

## IV. DISCUSSION

The results of this study clearly indicate the extreme susceptibility of sooty terns to VEE infection. Susceptibility has been demonstrated in other birds by the subcutaneous route of inoculation and by mosquito bite. However, these are the first data to indicate that terns are susceptible to VEE virus; more important, these are the first data indicating susceptibility of birds of any species by the respiratory route to predisseminated aerosols. This information, as indicated below, may be of primary importance in the field of epidemiology.

The most striking example of susceptibility was presented in Phase III of the study. One group of six birds was challenged with a measured respiratory dose of 39 $MICLD_{50}$ units inhaled. After the aerosol was allowed to decay naturally, a second group of six birds was challenged with an inhaled dose of less than one $MICLD_{50}$ as estimated by extrapolation from the earlier measurable cloud concentrations. Viremias were detected in four of the six birds in each dose group. In all infections, viremias were detected in the first blood sample, suggesting that chamber virus, and not later exposure to virus from infected birds, was the cause of infection.

The method of transmission has not been firmly established in the holding-line control birds, although it is strongly suspected that these birds were infected by the respiratory route. All testing and subsequent holding during incubation periods took place in a mosquito-free area, eliminating the possibility that a flying insect vector was involved. Further, no insects were found on the bird other than bird lice, and these do not normally feed on blood nor would they be expected to pass from one isolated live bird to another.

Although bleeding was accomplished in a common area, birds were handled on clean towels ringed with disinfectant. Dead birds were removed from the cages for post-mortem examination as soon as found. The cages were not reused without disinfection.

Excretion of virus in the feces has been demonstrated with Eastern equine encephalitis in pheasants.[10] With the assumption of this source of virus in the holding area, with the certainty that dust was created in the holding area, and with the demonstration of high susceptibility through inhalation, a bird-bird cycle has been strongly suggested.

Such a cycle of transmission requires a re-examination of the importance of birds as a natural reservoir of arboviruses. This is especially true when, as in this study, there were no obvious signs of illness from VEE virus infection. It seems likely, then, that normal living habits would not be seriously disrupted during an infection. One quite logical reason to date for rejection of birds as reservoirs has been the short

duration in the Temperate Zone of the bird-mosquito-bird cycle.[11] The data provided here suggest a cycle that can be operative through any season and depends only upon close association among birds.

The final transmission of VEE virus to other animals or to man has been suggested as a process employing a mosquito vector. The mosquito transmission cycle has been demonstrated previously with other species of birds but not, until this study, with sooty terns. The difference in response of A. aegypti and A. triseriatus mosquitoes to blood meals from infected hosts illustrates again the importance of the presence of the appropriate mosquito species before biological transmission can occur.

## V. SUMMARY

Sooty terns were found to be highly susceptible to infection with Venezuelan equine encephalitis virus by the respiratory route. The birds developed viremia within two days after challenge but failed to show histopathologic signs of infection or even obvious signs of illness. Viremias generally declined to negligible levels by the fifth day after challenge. The demonstration of viremia was accompanied by a significant rise in serum neutralization index 21 days after challenge. The ability of neutralizing antibodies to protect against subsequent challenge has not been studied.

Infections in many of the control birds in the common housing area indicated the strong possibility of a bird-bird transmission cycle without the participation of an insect vector. Thus, infection by inhalation was suggested. A bird-mosquito-bird transmission cycle was demonstrated with A. triseriatus mosquitoes but could not be demonstrated with A. aegypti.

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